Litton BIONETICS





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(1)

#3738

# MUTAGENICITY EVALUATION OF OTTO FUEL #2 IN THE AMES SALMONELLA/MICROSOME PLATE TEST

SEGMENT REPORT

N00014-78-C-0792

SUBMITTED TO:

CODE 4444
DEPT. OF NAVY
OFFICE OF NAVAL RESEARCH
ARLINGTON, VA. 22217

SUBMITTED BY:

LITTON BIONETICS, INC.
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LBI PROJECT NO. 20988

APRIL 1979

Litton BIONETICS

I. SPONSOR: U.S. Navy

II. MATERIAL

A. Identification: Otto Fuel #2

B. Date Received: November 7, 1979

C. Physical Description: Orange liquid

III. TYPE OF ASSAY: Ames Salmonella/Microsome Plate Test

IV. PROTOCOL NO.: 401 (DMT-100)

V. RESULTS

The results of this assay are presented in Tables 1 and 2.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella and Saccharomyces indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

The compound was tested over a series of concentrations such that there was either quantitative or qualitative evidence of some chemically-induced physiological effects at the high dose level. The low dose in all cases was below a concentration that demonstrated any toxic effect. The dose range employed for the evaluation of this compound was from 0.005 µl to 10 µl per plate. The compound was toxic to all the strains except TA-1538 and D4 at 5 µl and 10 µl per plate. Toxicity was also observed with the strain TA-1535 at 1 µl per plate.

The results of the tests conducted on the compound in the absence of a metabolic system were all negative.

The results of the tests conducted on the compound in the presence of the rat liver activation system were all negative. The test was repeated with TA-100 at the three lowest doses and intermediate doses of 0.05 µl and 0.5 µl per plate because of the increase in the number of revertants observed in the initial test. The repeat tests were also negative.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test compound, Otto Fuel #2, did not demonstrate mutagenic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions.

Submitted by:

Study Director

D.R. Jagannath, Ph.D. — Date: 4.20.79

Section Chief
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Reviewed by:

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Director
Department of Genetics
and Cell Biology

V. RESULTS

TABLE I

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: OTTO FUEL #2
B. SOLVENT: DMSO
C. TEST INITIATION DATE: 01/04/79
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

| TEST | SPECIES | TISSUE | REVERTANTS PER PLATE | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TA-1535 | | TA-1537 | | TA-1538 | | TA-98 | | TA-100 | | D4* | |
| NONACTIVATION | | | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| SOLVENT CONTROL | --- | --- | 15 | | 20 | | 19 | | 31 | | 154 | | 80 | |
| POSITIVE CONTROL** | --- | --- | 984 | | 924 | | 1609 | | 1643 | | 881 | | 423 | |
| TEST COMPOUND | | | | | | | | | | | | | | |
| 0.005000 UL | --- | --- | 16 | | 9 | | 12 | | 22 | | 215 | | 61 | |
| 0.010000 UL | --- | --- | 12 | | 13 | | 7 | | 30 | | 253 | | 72 | |
| 0.100000 UL | --- | --- | 15 | | 17 | | 10 | | 29 | | 238 | | 54 | |
| 1.000000 UL | --- | --- | 3 | | 22 | | 28 | | 27 | | 155 | | 72 | |
| 5.000000 UL | --- | --- | 0 | | 0 | | 11 | | 0 | | 0 | | 81 | |
| 10.000000 UL | --- | --- | 0 | | 0 | | 5 | | 0 | | 0 | | 76 | |
| ACTIVATION | | | | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 8 | | 22 | | 14 | | 40 | | 165 | | 165 | |
| POSITIVE CONTROL*** | RAT | LIVER | 598 | | 421 | | 1681 | | 2432 | | 1021 | | 200+ | |
| TEST COMPOUND | | | | | | | | | | | | | | |
| 0.005000 UL | RAT | LIVER | 16 | | 24 | | 13 | | 36 | | 229 | | 113 | |
| 0.010000 UL | RAT | LIVER | 16 | | 25 | | 11 | | 40 | | 233 | | 135 | |
| 0.100000 UL | RAT | LIVER | 10 | | 22 | | 7 | | 28 | | 242 | | 133 | |
| 1.000000 UL | RAT | LIVER | 18 | | 14 | | 8 | | 32 | | 170 | | 152 | |
| 5.000000 UL | RAT | LIVER | 0 | | 0 | | 13 | | 0 | | 0 | | 149 | |
| 10.000000 UL | RAT | LIVER | 0 | | 0 | | 14 | | 0 | | 0 | | 171 | |

* TRY+ REVERTANTS PER PLATE

** TA-1535 SODIUM AZIDE 1 UG/PLATE
TA-1537 9-AMINOACRIDINE 50 UG/PLATE
TA-1538 2-NITROFLUORENE 10 UG/PLATE
TA-98 2-NITROFLUORENE 10 UG/PLATE
TA-100 SODIUM AZIDE 1 UG/PLATE
D4 N-METHYL,N-NITRO,N-NITROSOGUANIDINE 10 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

*** TA-1535 2-ANTHRAMINE 2.5 UG/PLATE
TA-1537 2-ANTHRAMINE 2.5 UG/PLATE
TA-1538 2-ANTHRAMINE 2.5 UG/PLATE
TA-98 2-ANTHRAMINE 2.5 UG/PLATE
TA-100 2-ANTHRAMINE 2.5 UG/PLATE
D4 2-ANTHRAMINE 2.5 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

+ THERE IS NO KNOWN POSITIVE CONTROL COMPOUND THAT WORKS CONSISTENTLY WITH THIS STRAIN IN THE ACTIVATION PLATE ASSAYS.

V. RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: OTTO FUEL #2
B. SOLVENT: DMSO
C. TEST INITIATION DATE: 01/23/79
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

| TEST | SPECIES | TISSUE | REVERTANTS PER PLATE TA-100 | |
|---|---|---|---|---|
| | | | 1 | 2 |
| NONACTIVATION | | | | |
| SOLVENT CONTROL | --- | --- | 173 | |
| POSITIVE CONTROL** | --- | --- | 1367 | |
| TEST COMPOUND | | | | |
| 0.005000 UL | --- | --- | 145 | |
| 0.010000 UL | --- | --- | 182 | |
| 0.050000 UL | --- | --- | 178 | |
| 0.100000 UL | --- | --- | 159 | |
| 0.500000 UL | --- | --- | 155 | |
| ACTIVATION | | | | |
| SOLVENT CONTROL | RAT | LIVER | 146 | |
| POSITIVE CONTROL*** | RAT | LIVER | 1749 | |
| TEST COMPOUND | | | | |
| 0.005000 UL | RAT | LIVER | 157 | |
| 0.010000 UL | RAT | LIVER | 193 | |
| 0.050000 UL | RAT | LIVER | 218 | |
| 0.100000 UL | RAT | LIVER | 170 | |
| 0.500000 UL | RAT | LIVER | 241 | |

** TA-100 SODIUM AZIDE 1 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

*** TA-100 2-ANTHRAMINE 2.5 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for genetic activity in a microbial assay with and without the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Indicator Microorganisms

| | |
|---|---|
| Salmonella typhimurium | TA-1535<br>TA-1537<br>TA-1538<br>TA-98<br>TA-100 |
| Saccharomyces cerevisiae | D4 |

B. Activation System

1. Reaction Mixture

| Component | Final Concentration/ml |
|---|---|
| TPN (Sodium salt) | 4 µmol |
| Glucose-6-phosphate | 5 µmol |
| Sodium phosphate (dibasic) | 100 µmol |
| $MgCl_2$ | 8 µmol |
| KCl | 33 µmol |
| Homogenate S9 fraction | 0.1 ml |

2. S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 lot # B10-82 was purchased from Biological Products, Litton Bionetics, Inc. and was used in these assays.

2. MATERIALS (Continued)

C. Positive Control Chemicals

The chemicals used for positive controls in the nonactivation and activation assays are given in Tables 1 and 2 of Section V. Results.

D. Solvent

The solvent employed to prepare the stock solution of the test chemical is given in Tables 1 and 2 of Section V. Results. All dilutions of the test chemical were made using this solvent.

3. EXPERIMENTAL DESIGN

A. Plate Test (Agar Incorporation)

Approximately $10^8$ cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml molten agar supplemented with biotin and a trace of histidine. For nonactivation tests, at least 4 dose levels of the test compound were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests, at least 4 dose levels of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hours at 37°C and scored for the number of colonies growing on each plate. The concentrations of all chemicals are given in Tables 1 and 2 of Section V. Results. Positive controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

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* Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames agar incorporation method. Some dialkyl nitrosamines and certain substituted hydrazines are mutagenic in suspension assays, but not in the plate assay. Chemicals of these classes should be screened in a suspension assay.

3. EXPERIMENTAL DESIGN (Continued)

B. Recording and Presenting Data

The numbers of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

4. EVALUATION CRITERIA

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test chemical and the cells are incubated in the overlay for 2 days, and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the compound and the cells in the overlay permits constant exposure of the indicator cells for 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plates. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over 2 or 3 log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected.

4. EVALUATION CRITERIA (Continued)

B. Dose-Response Phenomena

Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

1. Strains TA-1535, TA-1537 and TA-1538

   If the solvent control value is within the normal range, a chemical that produces a positive dose response over three doses with the highest increase equal to three times the solvent control value is considered to be mutagenic.

2. Strains TA-98, TA-100 and D4

   If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-100 and 2-3 times the solvent control value for strains TA-98 and D4 is considered to be mutagenic. For these strains, the dose-response increase should start at approximately the solvent control value.

4. EVALUATION CRITERIA (Continued)

D. Evaluation Criteria for Ames Assay

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. It is also anticipated that if a given strain, e.g., TA-1537, responds to a mutagen in nonactivation tests, it will generally do so in activation tests (the converse of this relationship is not expected). While similar response patterns are not required for all mutagens, they can be used to enhance the reliability of an evaluation decision.

4. Reproducibility

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relationship Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Test is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relationships have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann et al. (1975) show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data presented in this report are based only on the demonstration, or lack, of mutagenic activity.

## REFERENCES

Ames, B.N., McCann, J. and Yamasake, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. 72, 5135-5139.

LBI ASSAY NO. 3738

LBI SAFETY NO. 3304

# MUTAGENICITY EVALUATION OF

# OTTO FUEL #2

# IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY

## FINAL REPORT

SUBMITTED TO:

DEPARTMENT OF THE NAVY
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ARLINGTON, VA. 22217

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
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LBI PROJECT NO. 20989

REPORT DATE: FEBRUARY 1979

Litton BIONETICS

## PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.

I. SPONSOR: Department of the Navy

II. MATERIAL (TEST COMPOUND): LBI ASSAY NUMBER #3738

A. Identification: Otto Fuel #2

B. Date Received: November 7, 1978

C. Physical Description: Orange-red liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. PROTOCOL NUMBER: 431 (DMT-106)

V. STUDY DATES:

A. Initiation: November 30, 1978

B. Completion: January 26, 1979

VI. SUPERVISORY PERSONNEL:

A. Study Director: Brian Myhr, Ph.D.

B. Laboratory Supervisor: Marie McKeon

VII. RESULTS:

The data are presented in Tables 1 and 2 on pages 4 and 5.

VIII. INTERPRETATION OF RESULTS:

The test compound, Otto Fuel #2, was dissolved in DMSO at a concentration of 333 µl/ml for use in the preliminary cytotoxicity test. Dilutions were performed with DMSO prior to final 1:100 dilutions into growth medium to obtain an applied concentration range of 3.3 µl/ml to 0.0079 µl/ml. The compound precipitated at concentrations near 1 µl/ml and above, forming a yellow film on the plastic culture tube, but most of the material appeared to redissolve during the 4 hour exposure period. The compound destroyed all the cells at 1.65 µl/ml within 24 hours of treatment. Therefore, the mutation assay was initiated with an applied concentration range of 4.0 µl/ml to 0.0313 µl/ml.

Two trials of the mutation assay were performed because a low average absolute cloning efficiency of about 54% was obtained for the negative (solvent and untreated) controls in the first assay (Table 1). Assays with cloning efficiencies above 50% are usually acceptable as supporting data for those in the optimum region above 70%. In this case the first assay was internally consistent and the positive control mutant frequencies were normal, so the test data was considered contributory.

VIII. INTERPRETATION OF RESULTS (continued):

The applied concentration range was the same for both trials; the five or six dose levels chosen for completion of the assays were selected to span the observed ranges of toxicity to growth with emphasis on the most toxic doses. After the cells were seeded for mutant selection and measurement of viability, the percent relative growth in the treated cultures was found to range from 87.5% to 7.0% without activation and from 93.6% to 0.7% with activation, both trials inclusive.

The results of the two mutation assays are presented in Tables 1 and 2.

Without activation, the mutant frequencies in the treated cultures in the first trial (Table 1) were comparable to the background frequency (average of the solvent and untreated negative controls) at all assayed doses from 1.5 µl/ml to 0.0625 µl/ml. A 2.5-fold increase over background is considered necessary to demonstrate mutagenesis at any given dose level. The 1.5 µl/ml dose was highly toxic and reduced the percent relative growth to 10.2%. In the second trial (Table 2), a 4.1-fold increase in mutant frequency was observed at 1.5 µl/ml, which resulted in a 7.9% relative growth. At the next lowest dose of 1.0 µl/ml, the percent relative growth was 12.2% and the mutant frequency was not significantly elevated over the background. These results suggest the compound becomes mutagenic under nonactivation conditions only for highly toxic treatments that reduce the percent relative growth below about 10%. Since the toxicity associated with a given dose level can vary from one trial to the next, the mutagenic activity may begin at 1.5 nl/ml or a higher applied concentration.

With activation, the test compound induced higher mutant frequencies at lower concentrations than under nonactivation conditions. In the first trial (Table 1), a 3.5-fold increase was induced by 0.25 µl/ml and a 3.7-fold increase occurred for the 0.50 µl/ml treatment. Similarly, a 2.9-fold increase in mutant frequency was obtained for 0.375 µl/ml in the second trial (Table 2). The two highest concentrations of 0.5 µl/ml and 0.75 µl/ml were extremely toxic and induced large increases in mutant frequency. In both cases, an insufficient number of cells was available to sample the usual population size ($3 \times 10^6$ cells) for mutants. Only $1.2 \times 10^6$ cells were analyzed at 0.5 µl/ml and 120 cells seeded for viable colony counts. For the culture exposed to 0.75 µl/ml, twice as many cells ($2.4 \times 10^6$) were analyzed for mutants and 240 cells were seeded for the viable colony count. The low sample sizes can result in increased variability and may be partly responsible for the 24-fold increase in mutant frequency observed at 0.50 µl/ml. The test compound clearly became strongly mutagenic for extremely toxic treatments.

VIII. INTERPRETATION OF RESULTS (continued):

The cloning efficiency in the second trial was 70% without activation and 90% with activation, which demonstrates good culturing conditions for the assay. For both trials, the average background frequencies were within the normal ranges for activation and nonactivation assays, and the positive control compounds yielded normal frequencies that were greatly in excess of the backgrounds. Thus, the mutation assays were considered to provide valid results.

IX. CONCLUSIONS:

The test compound, Otto Fuel #2, induced a significant increase in mutations at the TK locus in L5178Y mouse lymphoma cells at applied concentrations of 1.5 µl/ml without activation and from 0.25 µl/ml to 0.75 µl/ml with microsomal activation. These treatments were highly toxic.

Therefore, the test compound is considered to be active in the Mouse Lymphoma Forward Mutation Assay.

Submitted by:

Study Director

Brian Myhr 2-12-79

Brian Myhr, Ph.D. date
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Mammalian Genetics
Department of Genetics
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Reviewed by:

David J. Brusick 2/13/79

David J. Brusick, Ph.D. date
Director
Department of Genetics
and Cell Biology

Litton BIONETICS

## 4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS — TABLE I

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: OTTO FUEL #2
B. LSI CODE #: 3738
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 12/27/78

| TEST | S-9 SOURCE | S-9 TISSUE | DAILY COUNTS (CELLS/ML X 10E5) 1 | 2 | 3 | RELATIVE SUSPENSION GROWTH (% OF CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH* | MUTANT FREQUENCY** (X 10E+6) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **NONACTIVATION** | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 17.6 | 9.2 | | 100.0 | 75.0 | 173.0 | 100.0 | 100.0 | 43.4 |
| SOLVENT CONTROL | --- | --- | 15.4 | 12.0 | | 100.0 | 68.0 | 153.0 | 100.0 | 100.0 | 44.4 |
| UNTREATED CONTROL | --- | --- | 13.2 | 11.6 | | 88.3 | 65.0 | 164.0 | 100.6 | 88.9 | 39.6 |
| EMS .5 UL/ML | --- | --- | 5.9 | 11.6 | | 38.8 | 619.0 | 74.0 | 45.4 | 17.6 | 836.5 |
| TEST COMPOUND | | | | | | | | | | | |
| 0.062500 UL/ML | --- | --- | 13.0 | 10.6 | | 79.5 | 69.0 | 145.0 | 89.0 | 70.7 | 47.6 |
| 0.250000 UL/ML | --- | --- | 11.4 | 6.8 | | 44.7 | 78.0 | 319.0 | 195.7 | 87.5 | 24.5 |
| 0.500000 UL/ML | --- | --- | 10.6 | 10.0 | | 61.1 | 66.0 | 181.0 | 111.0 | 67.9 | 36.5 |
| 0.750000 UL/ML | --- | --- | 6.6 | 8.6 | | 32.7 | 84.0 | 162.0 | 99.4 | 32.5 | 51.9 |
| 1.000000 UL/ML | --- | --- | 7.4 | 6.2 | | 26.5 | 92.0 | 153.0 | 93.9 | 24.8 | 60.1 |
| 1.500000 UL/ML | --- | --- | 1.6 | 5.8 | | 10.0 | 104.0 | 165.0 | 101.2 | 10.2 | 63.0 |
| **ACTIVATION** | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 11.0 | 12.6 | | 100.0 | 83.0 | 171.0 | 100.0 | 100.0 | 48.5 |
| SOLVENT CONTROL | RAT | LIVER | 8.4 | 10.0 | | 100.0 | 80.0 | 152.0 | 100.0 | 100.0 | 52.6 |
| UNTREATED CONTROL | RAT | LIVER | 9.2 | 11.0 | | 90.9 | 108.0 | 154.0 | 95.4 | 86.7 | 70.1 |
| DMN .3 UL/ML | RAT | LIVER | 3.2 | 8.8 | | 23.7 | 268.0 | 33.0 | 20.4 | 4.8 | 812.1 |
| TEST COMPOUND | | | | | | | | | | | |
| 0.031300 UL/ML | RAT | LIVER | 11.8 | 9.4 | | 99.7 | 93.0 | 143.0 | 88.5 | 88.2 | 65.0 |
| 0.062500 UL/ML | RAT | LIVER | 10.4 | 9.2 | | 86.0 | 100.0 | 143.0 | 88.5 | 76.1 | 69.9 |
| 0.125000 UL/ML | RAT | LIVER | 6.4 | 16.0 | | 92.0 | 104.0 | 108.0 | 66.9 | 61.5 | 96.3 |
| 0.250000 UL/ML | RAT | LIVER | 7.0 | 12.0 | | 75.5 | 194.0 | 98.0 | 60.7 | 45.8 | 198.0 |
| 0.500000 UL/ML | RAT | LIVER | 2.4 | 7.0 | | 18.9 | 228.0+ | 107.0 | 66.3 | 12.5 | 213.1 |

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+6.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-6.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

+ = ONE PLATE CONTAMINATED; VALUE BASED ON TWO PLATES ONLY.

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: UTIO FUEL #2
B. LBL CODE #: 3738
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 01/14/79

| TEST | S-9 SOURCE | S-9 TISSUE | DAILY COUNTS (CELLS/ML X 10E5) 1 | 2 | 3 | RELATIVE SUSPENSION GROWTH (% OF CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH* | MUTANT FREQUENCY** (X 10E-6) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NONACTIVATION | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 19.0 | 12.4 | | 100.0 | 45.0 | 151.0 | 100.0 | 100.0 | 29.8 |
| SOLVENT CONTROL | --- | --- | 13.2 | 12.6 | | 100.0 | 41.0 | 167.0 | 100.0 | 100.0 | 24.6 |
| UNTREATED CONTROL | --- | --- | 15.8 | 8.0 | | 62.9 | 63.0 | 311.0 | 195.6 | 123.0 | 20.3 |
| EMS .5 UL/ML | --- | --- | 12.8 | 4.0 | | 25.5 | 489.0+ | 56.0 | 35.2 | 9.0 | 873.2 |
| TEST COMPOUND | | | | | | | | | | | |
| 0.031300 UL/ML | --- | --- | 14.2 | 7.6 | | 53.7 | 78.0 | C | ---- | ----- | ------ |
| 0.1 3000 UL/ML | --- | --- | 10.0 | 6.2 | | 30.9 | 70.0 | 243.0 | 152.8 | 47.2 | 28.8 |
| 0.750000 UL/ML | --- | --- | 7.0 | 4.6 | | 16.0 | 66.0 | 331.0 | 209.2 | 33.4 | 19.9 |
| 1.000000 UL/ML | --- | --- | 5.6 | 3.4 | | 9.5 | 109.0 | 204.0 | 113.2 | 10.8 | 53.4 |
| 1.500000 UL/ML | --- | --- | 4.6 | 3.4 | | 7.8 | 166.0 | 162.0 | 89.9 | 7.0 | 102.5 |
| ACTIVATION | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 12.2 | 10.6 | | 100.0 | 64.0 | 192.0 | 100.0 | 100.0 | 33.3 |
| SOLVENT CONTROL | RAT | LIVER | 15.0 | 9.6 | | 100.0 | 50.0 | 351.0+ | 100.0 | 100.0 | 14.2 |
| UNTREATED CONTROL | RAT | LIVER | 13.4 | 12.2 | | 85.3 | 66.0 | 268.0 | 98.7 | 84.2 | 24.6 |
| DMN .3 UL/ML | RAT | LIVER | 10.8 | 5.4 | | 30.4 | 280.0 | 43.0 | 15.8 | 4.8 | 651.2 |
| TEST COMPOUND | | | | | | | | | | | |
| 0.031300 UL/ML | RAT | LIVER | 15.0 | 10.4 | | 81.4 | 106.0 | 312.0 | 114.9 | 93.6 | 34.0 |
| 0.125000 UL/ML | RAT | LIVER | 8.8 | 6.8 | | 31.2 | 93.0 | 278.0 | 102.4 | 32.0 | 33.5 |
| 0.250000 UL/ML | RAT | LIVER | 8.4 | 6.0 | | 26.3 | 117.0 | 205.0 | 75.5 | 19.9 | 57.1 |
| 0.375000 UL/ML | RAT | LIVER | 7.6 | 7.0 | | 27.8 | 116.0 | 168.0 | 61.9 | 17.2 | 69.0 |
| 0.500000 UL/ML | RAT | LIVER | 0.5 | 1.2++ | | 1.9 | 242.0 | 42.0+ | 38.7 | 0.7 | 576.2 |
| 0.750000 UL/ML | RAT | LIVER | 1.0 | 2.4++ | | 3.8 | 263.0 | 93.0 | 42.8 | 1.6 | 282.8 |

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100

** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4. THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4. THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

\+ = ONE PLATE CONTAMINATED; VALUE BASED ON TWO PLATES ONLY.

C = CONTAMINATED (TWO OR MORE PLATES).

++ = INSUFFICIENT CELLS TO ASSAY 3 x $10^6$ CELLS FOR MUTANTS. SEE EVALUATION.

## 1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU).

## 2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

## 3. MATERIALS

### A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

### B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 50 or 100 µg/ml of BrdU.

3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 µl/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 µl/ml as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only five or six of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.

B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, $3 \times 10^6$ cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 µl S9/ml.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. Induction with Aroclor 1254 or other agents is performed by injections five days prior to sacrifice. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25M sucrose buffered with Tris at pH 7.4. When an adequate number of livers is obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.

EVALUATION CRITERIA

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 5 dose levels employed.
- The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

6. REFERENCE

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res., 31:17-29, 1975.

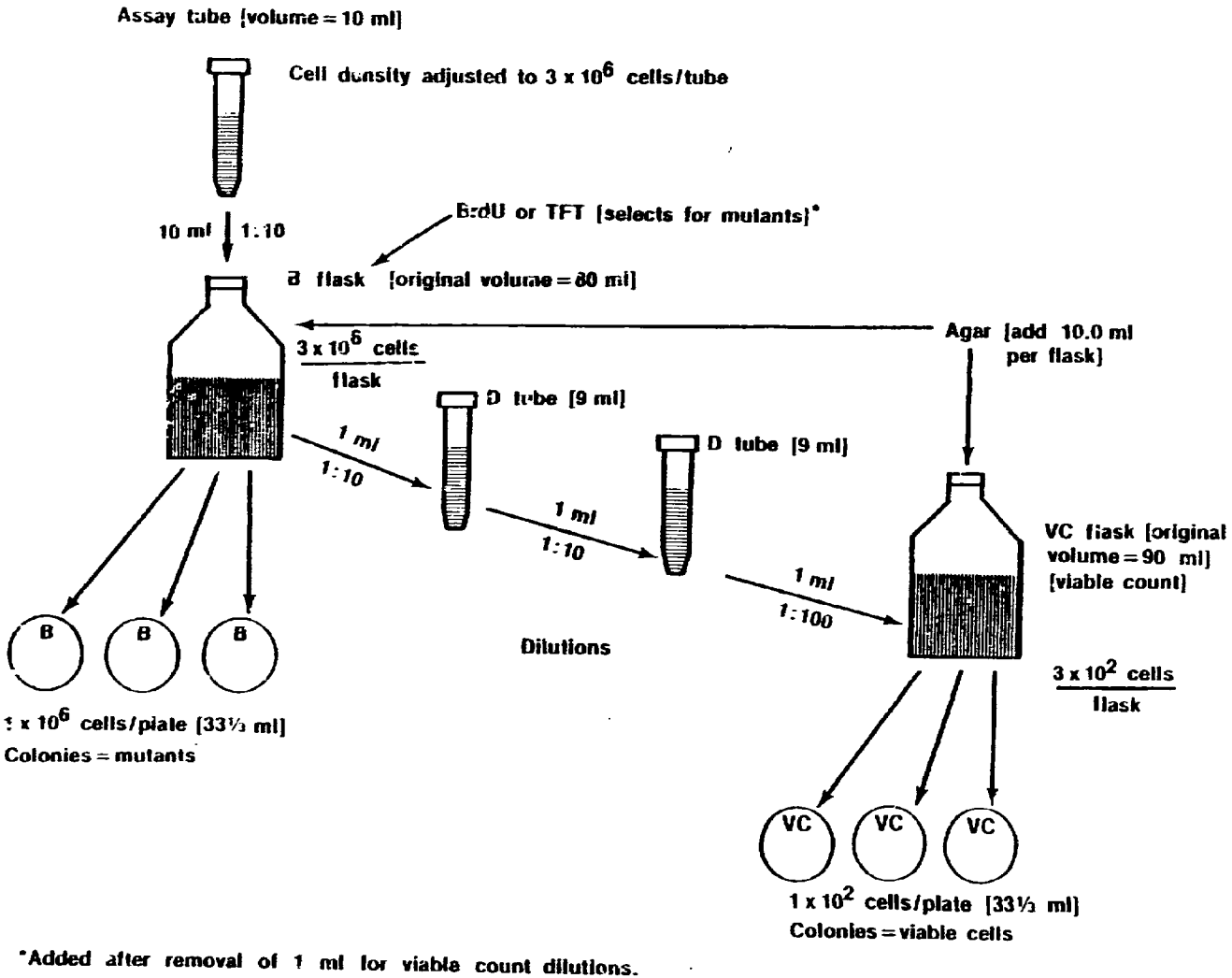



FIGURE 1. LYMPHOMA CLONING FLOW CHART

# MUTAGENICITY EVALUATION OF

# OTTO FUEL #2

# IN THE
# SISTER CHROMATID EXCHANGE ASSAY
# IN L5178Y MOUSE LYMPHOMA CELLS

## SEGMENT REPORT

**SUBMITTED TO:**

U.S. NAVY
800 N. QUINCY STREET
ARLINGTON, VA. 22217

**SUBMITTED BY:**

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO: 20990

MARCH 1979

Litton BIONETICS

# PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-VIII. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study. Item VI identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VII. Item VIII provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland, 20795.

Copies of the raw data will be supplied to the sponsor upon request. Copies of raw data are provided in the appendix.

I. SPONSOR: U.S. Navy

II. MATERIAL TESTED

A. Client's Identification: Otto Fuel #2

B. Genetic's Assay No.: 3738

C. Date Received: November 7, 1978

D. Physical Description:

III. TYPE OF ASSAY: Sister Chromatid Exchange Assay

IV. PROTOCOL NO.: 433

V. STUDY DATES:

A. Initiation Date: January 16, 1979

B. Completion Date: February 7, 1979

VI. RESULTS

The results of this assay are presented in Tables 1 and 2.

VII. INTERPRETATION OF RESULTS:

The test material, Otto Fuel #2, was tested for its ability to induce SCEs when used directly and also when used in the presence of a metabolic activation system that contains liver microsomal enzymes from Aroclor-induced rats.

Stock solution of Otto Fuel #2 was prepared in DMSO at 0.75 ml/ml and serial dilutions were performed in the same solvent so that predetermined dose levels (see Protocol) could be achieved without exceeding a final concentration of solution (in medium) of 0.01 ml/ml.

This compound failed to induce significant increases in SCE frequency (compared to the solvent controls) at all dose levels tested, with and without metabolic activation (Tables 1 and 2). Positive results with DMN indicate that the S9 activation system was functional. Dose levels employed in the activation study were somewhat lower than those employed in the direct assay, due to the fact that the compound was slightly more toxic following metabolic activation.

VIII. CONCLUSIONS:

The test compound does not induce SCE either with or without metabolic activation under the conditions of this assay.

Submitted by:

Study Director

Daniel Stetka, Ph D. — Date 4/4/79
Section Leader
Animal Genetics and
Cytogenetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick, Ph.D. — Date 4/4/79
Director
Department of Genetics
and Cell Biology

Litton BIONETICS

TABLE 1

SCE FREQUENCIES IN CELLS EXPOSED TO OTTO FUEL #2

WITHOUT METABOLIC ACTIVATION

| Treatment | Dose | No. of Chromosomes + | No. of SCE's | SCE/Chromosome ±SE | SCE/Cell |
|---|---|---|---|---|---|
| Negative Control (Medium) | --- | 770 | 158 | 0.205 ± 0.016 | 8.21 |
| Positive Control (EMS) | 0.5 µl/ml | 777 | 1043 | 1.342 ± 0.042* | 53.69* |
| Solvent Control (DMSO) | 0.1 ml/tube | 760 | 173 | 0.228 ± 0.017 | 9.10 |
| Test Compound | | | | | |
| Otto Fuel #2 | 20.0 nl/ml | 759 | 166 | 0.219 ± 0.017 | 8.75 |
| Otto Fuel #2 | 39.0 nl/ml | 756 | 158 | 0.209 ± 0.017 | 8.36 |
| Otto Fuel #2 | 78.0 nl/ml | 783 | 209 | 0.267 ± 0.018 | 10.68 |
| Otto Fuel #2 | 156.0 nl/ml | 752 | 175 | 0.233 ± 0.018 | 9.31 |
| Otto Fuel #2 | 313.0 nl/ml | 758 | 207 | 0.273 ± 0.019 | 10.92 |

*Significantly greater than solvent control, $P < 0.01$ (t-test).

+20 cells scored for each treatment.

TABLE 2

SCE FREQUENCIES IN CELLS EXPOSED TO OTTO FUEL #2

WITH METABOLIC ACTIVATION

| Treatment | Dose | No. of Chromosomes + | No. of SCE's | SCE/Chromosome ±SE | SCE/Cell |
|---|---|---|---|---|---|
| Negative Control (Medium) | --- | 738 | 170 | 0.230 ± 0.018 | 9.21 |
| Positive Control (DMN) | 0.3 µl/ml | 727 | 720 | 0.990 ± 0.037* | 39.61* |
| Solvent Control (DMSO) | 0.1 ml/tube | 750 | 222 | 0.296 ± 0.020 | 11.84 |
| Test Compound | | | | | |
| Otto Fuel #2 | 5.0 nl/ml | 749 | 225 | 0.300 ± 0.020 | 12.01 |
| Otto Fuel #2 | 10.0 nl/ml | 765 | 195 | 0.255 ± 0.018 | 10.20 |
| Otto Fuel #2 | 20.0 nl/ml | 738 | 207 | 0.280 ± 0.019 | 11.22 |
| Otto Fuel #2 | 39.0 nl/ml | 758 | 235 | 0.310 ± 0.020 | 12.40 |
| Otto Fuel #2 | 78.0 nl/ml | 762 | 241 | 0.316 ± 0.020 | 12.65 |

*Significantly greater than solvent control, $P < 0.01$ (t-test).

+20 cells scored for each treatment.

PROTOCOL

1. OBJECTIVE

The objective of this study was to evaluate Otto Fuel #2 for Sister Chromatid Exchange (SCE) induction in L5178Y mouse lymphoma cells.

2. MATERIALS AND METHODS

A. Toxicity

The solubility, toxicity, and doses for all chemicals were determined prior to screening. The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of the cells induced by a four-hour exposure to the chemical followed by a 24-hour expression period in growth medium. A minimum of four doses was selected from the range of concentrations by using the highest dose that showed no loss in growth potential as the penultimate dose and by bracketing this with one higher dose and at least two lower doses.

B. Indicator Cells

The cells used in this study were derived from Fischer mouse lymphoma cell line L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BrdU) sensitive.

C. Media

The cells were maintained in Fischer's medium for leukemic cells of mice with 10% horse serum and sodium pyruvate.

D. Control Compounds

1. Negative Control

The solvent in which the test compound was prepared was used as the solvent or vehicle control and is designated as solvent control in the data table. The actual solvent is listed in Table 1 of Section V. Results. A negative control consisting of cells exposed to media only is also used in the assay.

2. MATERIALS AND METHODS (continued)

D. Control Compounds

2. Positive Control

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and used as a positive control for the nonactivation studies at a final concentration of 0.5 µl/ml.

Dimethylnitrosamine (DMN), which induces mutation by base-pair substitution and requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for activation studies at a final concentration of 0.3 µl/ml.

E. Cell Treatment

Mouse lymphoma cells (L5178Y) were treated as described below. The test compound was added to aliquots of 3 million cells in growth medium at the predetermined doses with or without an S-9 activation mixture and incubated at 37°C for 4 hours on a rocker. The incubation period was terminated by washing the cells twice with growth medium. BrdU (0.1 mM final concentration) was then added to the culture tubes and incubation was continued in the dark for 20 hours or two cell cycles. This permits BrdU to be incorporated into the DNA through two replication cycles so that sister chromatid exchanges may be detected.

Colcemid was added to a concentration of $2 \times 10^{-7}$M during the last 3 hours of incubation, and metaphase cells collected by centrifugation. Treated cells were harvested in 0.075 M KCL fixed in Carnoy's fixative and air-dried onto microscope slides.

Sister chromatid exchanges were visualized by staining with techniques described in Stetka et al (Mutat. Res. 51, 1978).

F. Activation System

1. S9 Mixture

| Component | Final Concentration/ml |
|---|---|
| NADP (Sodium salt) | 2.4 mg |
| Isocitric acid | 4.5 mg |
| Homogenate S9 fraction | 15 µl |

2. S9 Homogenate

A 9,000 x g supernatant was prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 five days prior to kill according to the procedure of Ames et al. (1975). S9 samples were coded by lot number and assayed for milligrams protein per milliliter and relative P448/P450 activity by methods described in LBI Technical Data on Rat Liver S9 Product.

3. RESULTS

The data presented in Tables 1A and 1B show the concentrations of the test compound employed and the number of SCE's per cell.

Interpretation of data is based on the relative increase in SCE with respect to dose compared to the spontaneous level. Statistical analysis of the data is made by a t-statistic.

## REFERENCES

Ames, B.N., McCann, J. and Yamasake, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364.

GENETICS ASSAY #3738

# MUTAGENICITY EVALUATION OF

# OTTO FUEL

# IN THE
# MOUSE BONE MARROW
# CYTOGENETIC ANALYSIS

## SEGMENT REPORT

SUBMITTED TO:

U.S NAVY
800 N. QUINCY STREET
ARLINGTON, VA. 22217

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 21022

MARCH 1979

Litton BIONETICS

## PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-VI. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens."

Item V identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation of the results and conclusions are in Item VI.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed inconducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.

I. SPONSOR: U. S. Navy

II. MATERIAL

A. Identification: Otto Fuel

B. Date Received: November 7, 1978

C. Physical Description: Orange-red liquid

III. TYPE OF ASSAY: Mouse Bone Marrow Cytogenetic Analysis*

IV. PROTOCOL NO.: DMT-112

V. RESULTS

The toxicology and dosage selection results are presented in Table 1. The acute and subchronic test results have been collected from raw data sheets and tabulated in summary form in Table 2.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound, Otto Fuel, was evaluated for its ability to induce chromosome aberrations in bone marrow cells of mice exposed either acutely or subchronically to various dose levels.

Preliminary range finding studies were conducted using a wide range of doses to determine the $LD_{50}$ for oral administration of this compound. Probit analysis indicated a value of 1.6 ml/kg, and so the highest dose selected for use in the cytogenetic assay was 0.16 ml/kg (or 1/10 $LD_{50}$, according to standard Litton protocols). Lower doses were selected as 1/3 and 1/10 of this high dose (Table 1).

Otto Fuel solutions were prepared at 0.025 ml/ml in corn oil and diluted, as required, using the same solvent so that final dose levels could be achieved with the injection of 0.2 ml/animal/ dose. Dosing schedules and general experimental design are outlined in Table 3.

Results of the cytogenetic assay are presented in Table 2. Positive control values (structural aberration frequencies and percent cells with aberrations) were elevated significantly compared to negative (solvent) controls. Otto Fuel, on the other hand, does not appear to be clastogenic under the conditions of this test. Aberration frequencies were within normal range at all dose levels. The appearance of one ring at 6 hours with 0.016 ml/kg, and two rings at 24 hours with 0.16 ml/kg is somewhat suggestive, as this type of aberration is quite rare in controls. In addition, the aberrant cell frequency (percent cells with aberrations) shows very weak but positive dose responses at 6 and 24 hour kill times.

*Initiation Date: February 12, 1979 Completion Date: March 15, 1979

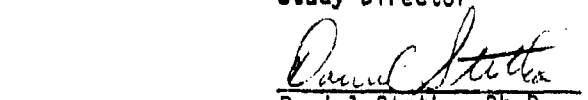
VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The actual increases are small, however, and do not by themselves constitute sufficient evidence for clastogenic activity of this compound. Considered together with the ring chromosome observation, these data do suggest that this compound might demonstrate significant clastogenic activity at higher (i.e., toxic) concentrations.

Overall, it must be concluded that unequivocal evidence for clastogenic activity of Otto Fuel was not obtained under the conditions of this assay.

Submitted by:

Study Director



Daniel Stetka, Ph.D. — 3/23/79 Date
Section Leader
Animal Genetic
and Cytogenetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick, Ph.D. — 3/23/79 Date
Director
Department of Genetics
and Cell Biology

Table 1

Toxicity and Dosage Information for Otto Fuel and Control Compounds

| | |
|---|---|
| Otto Fuel | $LD_{50}$ determined from range-finding study is 1.6 ml/kg per os (PO), leading to the following: |
| | High Dose (1/10 $LD_{50}$) = 0.16 ml/kg PO in corn oil (0.2 ml soln/mouse/dose) |
| | Inter Dose (1/30 $LD_{50}$) = 0.05 ml/kg PO in corn oil (0.2 ml soln/mouse/dose) |
| | Low Dose (1/100 $LD_{50}$) = 0.016 ml/kg PO in corn oil (0.2 ml soln/mouse/dose) |
| Negative Control* | Corn oil (the solvent vehicle), administered PO at 0.2 ml/mouse/dose |
| Positive Control* | TEM at 1 mg/kg administered PO in saline at 0.32 ml soln/mouse |

*The controls were shared with the bone marrow cytogenetic evaluation of Picramic Acid (U.S. Navy)

TABLE 2

A SUMMARY OF THE CYTOGENETIC ANALYSIS OF OTTO FUEL

| Treatment | Dose | Kill Time (Hrs)[a] | No. of Animals[b] | Total No. of Cells | Type and Number( ) of Aberrations Structural | Numerical | Structural[c] Aberration Frequency | No. of Cells with one or more Aberrations | Percent Cells with Aberrations |
|---|---|---|---|---|---|---|---|---|---|
| Negative Control | 0.2 ml Corn Oil | 6 | 8 | 400 | 2td,1f,2af(5) | 1h (1) | 0.013 | 6 | 1.5 |
| | | 24 | 7 | 228 | 1tb,1td,3f(5) | -- (0) | 0.022 | 5 | 2.2 |
| | | 48 | 6 | 227 | 1td,2f (3) | 2h (2) | 0.013 | 5 | 2.2 |
| | S.C. | 6 | 6 | 300 | 1th,4f,1af(6) | 4h (4) | 0.020 | 8 | 2.7 |
| Positive Control | 1.0 mg/kg | 24 | 5 | 175 | 13tb,23td 105f 17af,6t,4tr,1qr, 9pu+,3puc,2cr,2r 5d,38> (>228) | -- (0) | >1.303** | 107 | 61.7 |

[a]Time after final exposure when bone marrow was harvested

[b]Includes only those animals from which at least 5 scorable metaphases were obtained

[c]No. of Aberration/No. of Cells

*P<0.05 by t-test, two-tailed

** P<0.01 by t-test, two-tailed

TABLE 2

A SUMMARY OF THE CYTOGENETIC ANALYSIS OF OTTO FUEL

| Treatment | Dose | Kill Time (Hrs)[a] | No. of Animals[b] | Total No. of Cells | Type and Number( ) of Aberrations Structural | Numerical | Structural[c] Aberration Frequency | No. of Cells with one or more Aberrations | Percent Cells with Aberrations |
|---|---|---|---|---|---|---|---|---|---|
| Otto Fuel | 0.016 ml/kg | 6 | 8 | 385 | 3f, 1r (4) | 3h (3) | 0.010 | 7 | 1.8 |
| | | 24 | 7 | 314 | 3f, 1min (4) | 4h (4) | 0.013 | 7 | 2.2 |
| | | 48 | 7 | 299 | 1f (1) | 1h (1) | 0.003 | 2 | 0.7 |
| | S.C. | 6 | 8 | 344 | 1td,2f (4) 1af | 1h (1) | 0.012 | 5 | 1.5 |
| Otto Fuel | 0.05 ml/kg | 6 | 8 | 225 | 2td,1f (3) | 3h (3) | 0.013 | 6 | 2.7 |
| | | 24 | 6 | 271 | 2td,4f (6) | 5h (5) | 0.022 | 9 | 3.3 |
| | | 48 | 5 | 165 | 1f (1) | 1h (1) | 0.006 | 2 | 1.2 |
| | S.C. | 6 | 8 | 400 | 1tb,4f (5) | 2h (2) | 0.013 | 7 | 1.8 |
| Otto Fuel | 0.16 ml/kg | 6 | 7 | 248 | 1td,2f (6) 3af | 1h,1pp (2) | 0.024 | 8 | 3.2 |
| | | 24 | 8 | 348 | 5f,1af,(8) 2r | 4h (4) | 0.023 | 12 | 3.4 |
| | | 48 | 8 | 245 | 1f (1) | 4h (4) | 0.004 | 5 | 2.0 |
| | S.C. | 6 | 5 | 241 | 2f,2af (4) | 1h (1) | 0.017 | 5 | 2.1 |

[a] Time after final exposure when bone marrow was harvested

[b] Includes only those animals from which at least 5 scorable metaphases were obtained

[c] No. of Aberration/No. of Cells

*P<0.05 by t-test, two-tailed

** P<0.01 by t-test, two-tailed

ANY OF THE FOLLOWING ABBREVIATIONS MAY BE USED IN THE SUMMARY TABLES:

af = acentric fragment

cr = complex rearrangement

d = dicentric chromosome

f = fragment

h = hyperdiploid

min = minute chromosome

pp = polyploid

puc = pulverized cell

pu+ = pulverized chromosome

qr = quadriradial

r = ring

sb = chromosome break

sd = chromosome deletion

sg = chromosome gap

sl = slide lost or broken

t = translocation

tb = chromatid break

td = chromatid deletion

tg = chromatid gap

tr = triradial

> = greater than 10 aberrations

PROTOCOL

1. PURPOSE

The purpose of this study was to determine the potential genetic activity of a chemical administered to mice by examination of cells arrested at metaphase of mitosis for structural changes and rearrangements of their chromosomes.

2. MATERIALS

A. Animals

Adult, male mice (HA/ICR) purchased from Cumberland Labs were used in this cytogenetic study.

B. Control Chemicals

Triethylenemelamine (TEM) was used as the positive control compound. The negative control consisted of the solvent or vehicle used for the test compound. The concentrations and routes of administration are given in Table 1.

3. EXPERIMENTAL DESIGN

A. Animal Husbandry

The animals were group housed according to LBI standard operating procedures and offered a commercial diet (Purina) and water ad libitum unless contraindicated by the particular experimental design.

The animals were randomly assigned to experimental groups. Prior to study initiation, 10% of the animals were weighed and a mean body weight was determined for the group. Dose levels were established using this mean unless there was significant variation among individuals, in which case individual weighings and calculations were performed. Animals were identified by cage number.

3. EXPERIMENTAL DESIGN (continued)

B. Dosage Determination

Dosage information was calculated on the basis of range finding studies using 6 groups of 6 rats each. The high dose level was selected from these data. One-third and one-tenth of the high dose were used as the intermediate and low dose levels, respectively. For nontoxic compounds a maximum high dose level of 5 g/kg (or equivalent) is generally chosen.

4. METHODOLOGY

Table 3 shows the basic design of the test. Both acute (single dose) and subchronic (5 consecutive doses) sequences are provided. A total of 136 mice--104 in the acute study and 32 in the subchronic study--were used in the test as outlined in Table 3.

Two hours prior to kill, the animals were injected IP with 4.0 mg/kg colchicine. At times indicated in Table 3, mice were killed with $CO_2$ and the adhering soft tissue and epiphyses of one or both tibiae were removed. The marrow was aspirated from the bone and transferred to Hank's Balanced Salt Solution ( HBSS). The marrow button was collected by centrifugation and then resuspended in 0.075M KCL. The centrifugation was repeated and the pellet resuspended in Carnoy's fixative. The fixative was changed after one-half hour and the cells left overnight at 4°C.

Slides were prepared by dropping the cells from the fixative onto a glass slide and the film air-dried. Spreads were stained with 10% Giemsa at pH 6.8.

Slides were coded and scored for chromosomal aberrations. Where possible, 50 spreads were read for each animal dosed.

Animals which died during dosing were not replaced unless the number of deaths at a single dose level was 4 or more. In that case, the entire dose level was repeated following consultation with the sponsor.

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TABLE 3

MOUSE BONE MARROW CYTOGENETIC ANALYSIS
NUMBER OF ANIMALS USED

| Treatment | ACUTE STUDY Number of Animals Killed at 6, 24, and 48 Hr After Dosing | | | SUBCHRONIC STUDIES 5 Exposures-24 Hr Apart Animals Killed 6 Hr After Last Exposure | Total Animals |
|---|---|---|---|---|---|
| High Level | 6 Hr | 24 Hr | 48 Hr | 6 Hr | |
| High Level | 8 | 8 | 8 | 8 | 32 |
| Intermediate Level | 8 | 8 | 8 | 8 | 32 |
| Low level | 8 | 8 | 8 | 8 | 32 |
| Positive Control | - | 8 | - | - | 8 |
| Negative Control | 8 | 8 | 8 | 8 | 32 |

## 5. EVALUATION CRITERIA

A number of general guidelines has been established to serve as an aid in determining the meaning of bone marrow chromosomal aberrations.

### A. General

Basically, we were trying to establish whether a substance or its metabolites could interact with chromosomes to produce gross lesions or changes in chromosome numbers, and whether these were of a type which could survive more than one mitotic cycle of the cell. The assay design was such that bone marrow samples were taken at 6, 24, and 48 hrs afer an acute administration of the compound. Since the cell transit time for bone marrow is 20-24 hrs, one can, based on the time of kill, obtain an indication of when in the cell cycle a compound may be active.

### B. Aberrations/Records

All aberration figures detected by this assay resulted from breaks in the chromatin which either failed to repair or repaired in atypical combinations. We scored and recorded on standard forms gaps, breaks, fragments, and reunion figures which involved a single chromatid or both chromitids of a single chromosome. The number and type of aberration for each cell were recorded as was the number of chromosomes for every cell located and scored. Up to 50 cells were scored on each slide. Depending on the suitability of the material, it could have been necessary to prepare additional slides from the original fixed material. The location of cells bearing aberrations was identified by the use of coordinates on the mechanical stage.

### C. Data Interpretation

Data were summarized in tabular form and evaluated. Gaps were not counted as significant aberrations unless they were present at exceptionally high frequency. Open breaks were considered as indicators of genetic damage as were configurations resulting from the repair of breaks. The latter include translocations. multiradials, rings, multicentrics, etc. Reunion figures such as these were weighted slightly higher than breaks since they usually result from more than one break and may lead to stable configurations.

The number of aberrations per cell is also considered to be significant; cells with more than one aberration were considered to indicate more genetic damage than those containing evidence of single events. Consistent variations from the euploid number were also considered in the evaluation of mutagenic potential.

5. EVALUATION CRITERIA (continued)

C. Data Interpretation

Comparison with a concurrent negative control which lacks aberrations can suggest statistical significance; therefore, treatment data may also be considered against historical control data. In either event the type of aberration, its frequency, and its correlation to dose trends within a given time period, were all considered in evaluating a compound as being mutagenically positive or negative.

#3738

# MUTAGENICITY EVALUATION OF OTTO FUEL #2 IN THE MOUSE DOMINANT LETHAL ASSAY

SEGMENT REPORT

SUBMITTED TO:
CODE 4444
DEPT. OF NAVY
OFFICE OF NAVAL RESEARCH
ARLINGTON, VIRGINIA 22217

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 21021

MAY 1979

Litton BIONETICS

I. SPONSOR U.S. Navy

II. MATERIAL

A. Identification: Otto Fuel #2

B. Date Received: November 7, 1978

C. Physical Description: Yellow liquid

III. TYPE OF ASSAY: Mouse Dominant Lethal Assay

IV. STUDY DATES

A. Initiation Date: January 29, 1979

B. Completion Date: April 5, 1979

V. PTOTOCOL NO.: 470

VI. RESULTS

The results are presented in Tables 2 - 7. Table 1 provides treatment information. The remaining tables summarize the test results and statistical analyses.

VII. INTERPRETATION OF RESULTS AND CONCLUSIONS

Male mice were exposed to 0.016 ml/kg, 0.05 ml/kg and 0.16 ml/kg of Otto Fuel #2 and mated to virgin females over the entire spermatogenic cycle. Adminstration of the test material was by oral gavage. Detailed dosing information is provided in Table 1.

The results of the dominant lethal scoring are given in Tables 2-7 and cover all significant parameters of mating, fertility and fetal wastage.

Fertility-All fertility data were within the historical range and no compound related effects were observed.

Implants per pregnant female-All data were within the historical range and no compound related effects were observed.

Dead implants per pregnant female-The positive control was significantly increased in this parameter over weeks 1-4. All other data were within the historical range.

Females with one or more dead implants-The positive control values were slightly elevated compared to the negative control data. Only week 2 positive control results were significant. These data indicate a relatively uniform distribution of dead implants.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

Females with two or more dead implants-The positive control values for weeks 1-4 were highly significant indicating a strong response with TEM. All other test data were within the normal range. These data suggest a relatively uniform distribution of dead implants in the test groups.

Dead implants per total implants-Except for a single increase at 0.05 ml/kg in week 3 none of the test data were significant. TEM showed a positive dominant lethal effect in weeks 1, 2 and 3.

Otto Fuel #2 was not active in the dominant lethal assay conducted in mice.

Submitted by:

Study Director

_____ _____

Dan Stetka — Date
Section Leader
Animal Genetics
and Cytogenetics
Department of Genetics
and Cell Biology

Reviewed by:

_____ _____

David J. Brusick, Ph.D. — Date
Director
Department of Genetics
and Cell Biology

**Table 1**

**Sponsor** U.S. Navy **Study ID** Mouse Dominant Lethal **Initiation Date** January 29, 1979

**Project No.** 21021 **Strain/Species** CD-1 Mice **Termination Date** April 5, 1979

**Compound** Otto Fuel #2 **Breeder** Charles River **Location** Kensington

**Assay No.** 3738 **Purchase Order No.** 87857 **Room Nos.** L, 5X

| Treatment | Vehicle | Dosage[B] | Route of Administration | Volume/ Animal [ml] | Number of Administrations | Animal Numbers |
|---|---|---|---|---|---|---|
| Otto Fuel #2 | Corn Oil | 0.016 ml/kg | PO | 0.2 | 5 | 9863-9872 |
| Otto Fuel #2 | Corn Oil | 0.05 ml/kg | PO | 0.2 | 5 | 9873-9882 |
| Otto Fuel #2 | Corn Oil | 0.16 ml/kg | PO | 0.2 | 5 | 9883-9892 |
| Corn Oil | -- | 6.0 ml/kg | PO | 0.2 | 5 | 9893-9902 |
| TEM | 0.85% Saline | 0.35 mg/kg | IP | 0.12 | 1 | 9913-9922 |

Supervisory Personnel: Gary Roy

**A** Dosage levels based on client provided information.
**B** Dosage levels based on $LD_{50}$ determination.
**C** Toxic signs noted:

TABLE 2

COMPOUND: OTTO FUEL#2 FERTILITY INDEX 3733 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1600 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 581/1050 = 0.55 | 7/ 16 = 0.44 | 10/ 20 = 0.50 | 11/ 20 = 0.55 | 12/ 20 = 0.60 | 9/ 20 = 0.45 | | |
| 2 | 755/1090 = 0.69 | 12/ 16 = 0.75 | 14/ 20 = 0.70 | 13/ 20 = 0.65 | 13/ 20 = 0.65 | 14/ 20 = 0.70 | | |
| 3 | 715/1050 = 0.68 | 13/ 16 = 0.81 | 13/ 20 = 0.65 | 13/ 19 = 0.68 | 11/ 19 = 0.58 | 12/ 20 = 0.60 | | |
| 4 | 711/1052 = 0.68 | 12/ 16 = 0.75 | 16/ 20 = 0.80 | 13/ 20 = 0.65 | 17/ 20 = 0.85 | 17/ 20 = 0.85 | | |
| 5 | 661/1049 = 0.63 | 12/ 14 = 0.86 | 18/ 20 = 0.90 | 17/ 20 = 0.85 | 16/ 18 = 0.89 | 17/ 20 = 0.85 | | |
| 6 | 698/1050 = 0.66 | 6/ 16 = 0.38 | 18/ 20 = 0.90** | 14/ 20 = 0.70* | 10/ 18 = 0.56 | 9/ 20 = 0.45 | | |
| 7 | 655/1010 = 0.65 | 13/ 16 = 0.81 | 14/ 20 = 0.70 | 16/ 20 = 0.80 | 11/ 18 = 0.61 | 14/ 19 = 0.74 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 3

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

COMPOUND: OTTO FUEL#2 3738 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1600 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 6759/ 591 = 11.63 | 77/ 7 = 11.00 | 90/ 10 = 9.00 | 128/ 11 = 11.64 | 137/ 12 = 11.42 | [illegible]/ 9 = 12.67 | | |
| 2 | 8810/ 755 = 11.67 | 138/ 12 = 11.50 | 83/ 14 = 5.93** | 152/ 13 = 11.69 | 133/ 13 = 10.23 | 158/ 14 = 11.29 | | |
| 3 | 8522/ 715 = 11.92 | 138/ 13 = 10.62 | 154/ 13 = 11.85 | 167/ 13 = 12.85 | 116/ 11 = 10.55 | 153/ 12 = 12.75 | | |
| 4 | 9371/ 711 = 11.77 | 129/ 12 = 10.75 | 174/ 16 = 10.88 | 142/ 13 = 10.92 | 184/ 17 = 10.82 | 185/ 17 = 10.88 | | |
| 5 | 7787/ 661 = 11.79 | 146/ 12 = 12.17 | 199/ 18 = 11.06 | 211/ 17 = 12.41 | 192/ 16 = 12.00 | 197/ 17 = 11.59 | | |
| 6 | 8362/ 688 = 12.15 | 60/ 6 = 10.00 | 199/ 18 = 11.06 | 153/ 14 = 10.93 | 111/ 10 = 11.10 | 108/ 9 = 12.00 | | |
| 7 | 8023/ 655 = 12.24 | 170/ 13 = 13.08 | 171/ 14 = 12.21 | 189/ 16 = 11.81* | 130/ 11 = 11.82 | 171/ 14 = 12.21 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 4

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE
COMPOUND: OTTO FUEL #2 3738 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1600 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 392/ 591 = 0.67 | 8/ 7 = 1.14 | 54/ 10 = 5.40** | 9/ 11 = 0.82 | 13/ 12 = 1.08 | 10/ 9 = 1.11 | | |
| 2 | 637/ 755 = 0.84 | 10/ 12 = 0.83 | 58/ 14 = 4.14** | 10/ 13 = 0.77 | 12/ 13 = 0.92 | 12/ 14 = 0.86 | | |
| 3 | 544/ 715 = 0.76 | 7/ 13 = 0.54 | 27/ 13 = 2.08** | 9/ 13 = 0.69 | 22/ 11 = 2.00 | 5/ 12 = 0.42 | | |
| 4 | 553/ 711 = 0.78 | 5/ 12 = 0.42 | 26/ 16 = 1.63* | 14/ 13 = 1.08 | 8/ 17 = 0.47 | 21/ 17 = 1.24 | | |
| 5 | 410/ 661 = 0.62 | 5/ 12 = 0.42 | 13/ 18 = 0.72 | 12/ 17 = 0.71 | 13/ 16 = 0.81 | 9/ 17 = 0.53 | | |
| 6 | 516/ 688 = 0.75 | 3/ 6 = 0.50 | 8/ 18 = 0.44 | 5/ 14 = 0.36 | 8/ 10 = 0.80 | 4/ 9 = 0.44 | | |
| 7 | 398/ 655 = 0.60 | 9/ 13 = 0.69 | 5/ 14 = 0.36 | 17/ 16 = 1.06 | 3/ 11 = 0.27 | 8/ 14 = 0.57 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 5

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS
COMPOUND: OTTO FUEL#2 3738 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1500 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 297/ 531 = 0.44 | 5/ 7 = 0.71 | 10/ 10 = 1.00 | 6/ 11 = 0.55 | 7/ 12 = 0.58 | 6/ 9 = 0.67 | | |
| 2 | 289/ 755 = 0.38 | 7/ 12 = 0.58 | 14/ 14 = 1.00* | 5/ 13 = 0.38 | 7/ 13 = 0.54 | 8/ 14 = 0.57 | | |
| 3 | 332/ 715 = 0.46 | 6/ 13 = 0.46 | 10/ 13 = 0.77 | 5/ 13 = 0.38 | 9/ 11 = 0.82 | 3/ 12 = 0.25 | | |
| 4 | 368/ 711 = 0.49 | 5/ 12 = 0.42 | 10/ 16 = 0.63 | 7/ 13 = 0.54 | 6/ 17 = 0.35 | 9/ 17 = 0.53 | | |
| 5 | 259/ 661 = 0.39 | 4/ 12 = 0.33 | 7/ 18 = 0.39 | 9/ 17 = 0.53 | 10/ 16 = 0.63 | 7/ 17 = 0.41 | | |
| 6 | 326/ 699 = 0.47 | 3/ 6 = 0.50 | 6/ 18 = 0.33 | 4/ 14 = 0.29 | 5/ 10 = 0.50 | 4/ 9 = 0.44 | | |
| 7 | 279/ 655 = 0.43 | 7/ 13 = 0.54 | 5/ 14 = 0.36 | 5/ 16 = 0.31 | 3/ 11 = 0.27 | 5/ 14 = 0.36 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 6

PROPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS
COMPOUND: DIESEL FUEL #2 1738 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1600 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 109/ 581 = 0.17 | 2/ 7 = 0.29 | 8/ 10 = 0.8* | 2/ 11 = 0.18 | 3/ 12 = 0.25 | 3/ 9 = 0.33 | | |
| 2 | 136/ 755 = 0.18 | 3/ 12 = 0.25 | 11/ 14 = 0.79** | 3/ 13 = 0.23 | 4/ 13 = 0.31 | 3/ 14 = 0.21 | | |
| 3 | 114/ 715 = 0.16 | 1/ 13 = 0.08 | 9/ 13 = 0.69** | 2/ 13 = 0.15 | 3/ 11 = 0.27 | 1/ 12 = 0.08 | | |
| 4 | 123/ 711 = 0.17 | 0/ 12 = 0.0 | 7/ 16 = 0.44* | 4/ 13 = 0.31 | 2/ 17 = 0.12 | 4/ 17 = 0.24 | | |
| 5 | 92/ 661 = 0.14 | 1/ 12 = 0.08 | 3/ 18 = 0.17 | 3/ 17 = 0.19 | 3/ 16 = 0.19 | 2/ 17 = 0.12 | | |
| 6 | 108/ 648 = 0.16 | 0/ 6 = 0.0 | 2/ 18 = 0.11 | 1/ 14 = 0.07 | 3/ 10 = 0.30 | 0/ 9 = 0.0 | | |
| 7 | 79/ 655 = 0.12 | 2/ 13 = 0.15 | 0/ 14 = 0.0 | 2/ 14 = 0.13 | 0/ 11 = 0.0 | 3/ 14 = 0.21 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 7

DEAD IMPLANTS / TOTAL IMPLANTS

COMPOUND: DIESEL FUEL #2 1338 STUDY: SUBCHRONIC SPECIES: MICE

| WEEK | HIST. NEG. CONT. | NEG. CONTROL | POS. CONTROL | 0.0160 ML/KG | 0.0500 ML/KG | 0.1600 ML/KG | LOG DOSE | ARITH DOSE |
|---|---|---|---|---|---|---|---|---|
| 1 | 392/6759 = 0.06 | 6/ 77 = 0.10 | 54/ 90 = 0.60** | 9/128 = 0.07 | 13/137 = 0.09 | 10/114 = 0.09 | | |
| 2 | 637/8910 = 0.07 | 10/138 = 0.07 | 58/ 83 = 0.70** | 10/152 = 0.07 | 17/133 = 0.09 | 12/158 = 0.08 | | |
| 3 | 544/8522 = 0.06 | 7/139 = 0.05 | 27/154 = 0.18* | 9/167 = 0.05 | 22/116 = 0.19* | 5/153 = 0.03 | | |
| 4 | 553/8371 = 0.07 | 5/129 = 0.04 | 24/174 = 0.15 | 14/142 = 0.10 | 8/184 = 0.04 | 21/185 = 0.11 | | |
| 5 | 410/7787 = 0.05 | 5/146 = 0.03 | 13/199 = 0.07 | 12/211 = 0.06 | 13/192 = 0.07 | 9/197 = 0.05 | | |
| 6 | 516/8362 = 0.06 | 3/ 60 = 0.05 | 8/199 = 0.04 | 5/153 = 0.03 | 8/111 = 0.07 | 4/108 = 0.04 | | |
| 7 | 396/8020 = 0.05 | 9/170 = 0.05 | 5/171 = 0.03 | 17/189 = 0.09 | 3/130 = 0.02 | 8/171 = 0.05 | | |

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for its ability to induce dominant lethality in mice.

2. OVERVIEW AND RATIONALE

The dominant lethal assay is designed to determine the ability of a compound to induce genetic damage in the germ cells of treated male mice leading to fetal wastage. Chromosome aberrations including breaks, rearrangements, and deletions are believed to produce the dominant lethality although ploidy changes and chromosome nondisjunction may also be detected in this assay. Male mice are exposed to several dose levels of the test compound for 5 days and then mated over the entire period of spermatogenesis to unexposed virgin females. At mid-pregnancy, the females are killed and scored for the number of living and dead implants as well as the level of fertility. These results are then compared to data from control animals and used to determine the degree of induced dominant lethality.

Evidence of dominant lethality emphasizes that the compound is able to reach the developing germ cells and induce genetic damage. It also suggests, but does not measure directly, that in addition to the detected gross chromosomal lesions, more subtle balanced lesions or specific locus gene mutations may be produced. These latter types have a good chance of being transmitted to the gene pool of future offspring.

3. EXPERIMENTAL DESIGN

Ten (10) random bred, male mice from a closed colony were assigned to 1 of 5 groups. Three of these groups received different dose levels of the test compound; a fourth group received only the solvent or vehicle; and the fifth group received a known mutagen and served as the positive control group. The test compound and control compounds were administered as specified in Table 1. Triethylenemelamine (TEM) was used as the positive control and was given as a single intraperitoneal injection. Following treatment, each male was rested for 2 days and then caged with 2 unexposed virgin females on the third day. At the end of 5 days, these females were removed. This weekly mating sequence was continued for 7 weeks. Each pair of mated females was transferred to a fresh cage, and approximately 14 days after the midweek of being caged with the male, the females were killed with $CO_2$. At necropsy, their uteri were examined for dead and living implants, and total implantations. Animals which died during dosing were not replaced unless there was 75% mortality at a single dose level. In that case the compound toxicity was reviewed, and the entire dose level was repeated.

3. EXPERIMENTAL DESIGN (Continued)

A. Animals

Random bred, adult male and female mice, strain CD-1 were purchased from the Charles River Breeding Laboratories, Inc. Male and female mice were at least 8 weeks of age when purchased.

B. Animal Husbandry

Males were housed individually and females housed in pairs (except during mating) in shoe box cages on AB-SORB-DRI bedding.

All animals were quarantined prior to being used in the study to acclimate them to the new laboratory conditions. Purina Lab Chow was used as the basic diet food, and water were offered ad libitum. Light was provided on a 12-hour light/dark cycle.

Personnel handling animals or working within the animal facility wore suitable protective laboratory garments, including face masks or respirators.

C. Dosage Determination

Dosage information was calculated on the basis of range finding studies using at least 5 groups of 6 mice each. LD50, LD5, and LD1 concentrations were computer generated based on the preliminary study. The high dose level was selected from these data. One-third and one-tenth of the high dose were used as the intermediate and low dose levels, respectively.

D. Records

The number of dead and living implants, and total implantation sites were recorded on a standardized record form. Data were keypunched directly from these forms to computer entry cards, and analyzed for statistical significance as outlined in Appendix A. Original copies of all data are stored in the Litton Bionetics, Inc. archival system.

## 4. EVALUATION CRITERIA

Both pre- and post-implantation losses contribute to dominant lethality. The former is reflected in the total number of implantation sites per pregnant female and strictly measured by the difference between the number of corpora lutea gravidus and the number of implantation sites. Toxic or physiological effects on sperm may also reduce the number of implantation sites. Therefore, unless subtle physiological effects on sperm can be discounted, pre-implantation loss is not as rigorous and indication of dominant lethality as post-implantation loss. Corpora lutea cannot be reliably counted in mice and, therefore, pre-implantation loss is not evaluated in studies using mice. Post-implantation losses are measured as early and late fetal deaths plus the number of resorption sites.

Dominant lethality is typically determined from: a) a mutation index derived from the ratio of dead to total implants; or b) the number of dead implants per pregnant female. In interpreting these values it must be remembered that the former measurement reflects both pre- and post-implantation losses and that the ratio is affected by changes in either the numerator or the denominator. For this reason the second parameter is perhaps a better indicator of post-implantation loss. This becomes especially so if one concurrently examines the number of living embryos per pregnant female. The two sets of data should be inversely related. In other words, if true dominant lethality is being observed, then a significant increase in the number of dead implants per pregnant female should be accompanied by a significant decrease in the number of living implants per pregnant female.

These ratios are compared with both concurrent and historical control data for significant statistical differences. Dose-related trends are also looked for, but may not always be found. For example, some compounds such as EMS tested in mice show a threshold value and then a very steep rise. Certain portions of the response might be missed, depending on the spacing of the dose levels used.

True, as opposed to spurious, dominant lethality also tends to cluster according to the stage of spermatogenesis affected and typically would not be expected to appear in widely spaced weeks or blocks of weeks.

All data which are indicated as being statistically significant must also be strongly evaluated for their biological significance. By bringing both statistical and biological selective pressures to bear on the data gathered, an estimate of dominant lethality and of risk to the gene pool should be obtainable.

## REFERENCES

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# APPENDIX A

# STATISTICAL ANALYSIS

APPENDIX A

Analysis of Data

1. Fertility Index

a. The fertility index is defined as F.I. = # of pregnant females/ # of mated females. It is calculated for each week (in subacute study) or at the end of 8 weeks (in acute study) and for each dose level, negative control, and positive control.

b. A chi-square test is used to compare each treatment group and positive control to negative control.

$$\chi_i^2 = \frac{(N_0 + N_i)\,(\,n_0(N_i - n_i) - n_i(N_0 - n_0) - (N_0 + N_i)/2)^2}{(n_0 + n_i)(N_0 - n_0 + N_i - n_i)N_0N_i}$$

where

$n_i$ = # impregnated in i-th test group

$n_0$ = # impregnated in negative control group

$N_i$ = # of females mated in the i-th test group

$N_0$ = # of females mated in negative control group

A 2 x 2 table is formed as follows:

| | control | test |
|---|---|---|
| # impreg | $n_0$ | $n_i$ |
| # not impreg | $N_0 - n_0$ | $N_i - n_i$ |

Significance at the 5 and 1% levels is indicated with asterisks.

c. Armitage's trend for linear proportions is used to test whether the fertility index is linearly related to arithmetic or log dose.

The following table is set up:

| | -control | dose 1 | dose 2 | dose 3 | dose k | totals |
|---|---|---|---|---|---|---|
| # impreg | $n_0$ | $n_1$ | $n_2$ | $n_3$ | $n_k$ | t |
| # not impreg | $N_0 - n_0$ | $N_1 - n_1$ | $N_2 - n_2$ | $N_3 - n_3$ | $N_k - n_k$ | T - t |
| totals | $N_0$ | $N_1$ | $N_2$ | $N_3$ | $N_k$ | T |

and Armitage's chi-square is calculated:

$$\chi_A^2 = \chi_{(k-1)}^2 - \chi_1^2$$

where

$$\chi_1^2 = \frac{T(T\sum_{i=0}^{k} n_i x_i - t\sum_{i=0}^{k} N_i x_i)^2}{t(T-t)(T\sum_{i=0}^{k} N_i x_i^2 - (\sum_{i=0}^{k} N_i x_i)^2)}$$

$$\chi_{(k-1)}^2 = \frac{T^2(\sum_{i=0}^{k} n_i^2/N_i - t^2/T)}{t(T-t)}$$

and the $x_i$ are the dose levels. This calculation is repeated with x replaced by $\log_{10} x$. The 5 and 1% significance levels are indicated by dollar signs.

2. Total Number of Implantations

a. The total number of implantations is evaluated by the Student's t-test to determine whether the average number of implantations per pregnant female for each treatment group and the positive control group differs significantly from the negative control group.

$n_i$ = # of pregnant females at dose level i.
$u_{ij}$ = # of implantations for pregnant female j in dose group i.

$$\bar{u}_i = 1/n_i \left(\sum_{j=1}^{n_i} u_{ij}\right)$$

$$s_i^2 = \sum_{j=1}^{n_i} (u_{ij} - \bar{u}_i)^2$$

$$t_i = \bar{u}_0 - \bar{u}_i \Big/ \left(\frac{s_0^2 + s_i^2}{n_0 + n_i - 2} \left(\frac{1}{n_0} + \frac{1}{n_i}\right)\right)^{\frac{1}{2}}$$

$$d.f. = n_0 + n_i - 2$$

Significance at the 5 and 1% levels is indicated by asterisks.

b. A regression fit of the average number of implantations, $\bar{u}_i$, is made for both the arithmetic and logarithmic dose ($x_i$ and log $x_i$). The doses $x_i$ are used as independent variables and the fit includes data from the three treatment groups and the control group.

$N$ = total # of pregnant females in all groups.

$x_i$ = dose/log (dose) for the i-th female.

$U_i$ = # of implantations for the i-th female.

$$\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

$$SS_x = \sum_{i=1}^{N} (x_i - \bar{x})^2$$

$$\bar{U} = \frac{1}{N}\sum_{i=1}^{N} U_i$$

$$SS_u = \sum_{i=1}^{N} (U_i - \bar{U})^2$$

$$S_{xu} = \sum_{i=1}^{N} (x_i - \bar{x})(U_i - \bar{U})$$

$B$ = estimate of slope of regression line = $S_{xu}/SS_x$

$A$ = estimate of intercept of regression line = $\bar{U} - B\,\bar{x}$

VARU = variance of U about regression line

$$= \frac{SS_u - S_{xu}2/SS_x}{N-2}$$

VARB = variance of B = $\frac{VARU}{SS_x}$

VARA = variance of A = VARU $(\frac{1}{N} + \frac{\bar{x}^2}{SS_x})$

TB = $B/(VARB)^{\frac{1}{2}}$ = t-statistic for testing the hypothesis that the regression slope is zero

DF = N-2 = # of degrees of freedom for T B

CVUX = coefficient of variation of U about x

= $(VARU.X)^{\frac{1}{2}}/\bar{U}$

VARU.X = $\frac{1}{N-2}(SS_U - S_{XU}^2/SS_X)$

SDY = standard deviation of U about the regression line

= $(VARU.X)^{\frac{1}{2}}$

SDS = standard deviation of the slope

= $(VARB)^{\frac{1}{2}}$

SDA = standard deviation of intercept

= $(VARA)^{\frac{1}{2}}$

Significant difference of the slope from zero is indicated at the 5 and 1% levels.

3. Total Number of Corpora Lutea
(For rats only)

a. The average number of corpora lutea per pregnant female is evaluated by t-test to determine whether each treatment group differed significantly from the control group. Use the equation described in Step 2 above with

$u_{ij}$ = # of corpora lutea for pregnant female j in dose group i.

c. A regression fit of the average number of corpora lutea per pregnant female is made for both the arithmetic and logarithmic dose. Use the equations described in Step 2 above with

$u_i$ = # of corpora lutea for the i-th female

4. Preimplantation Losses
(For rats only)

a. The number of preimplantation losses is the number of corpora lutea minus the number of implantations.

$Y_{ij}$ = preimplantation losses for j-th female in i-th group

$V_{ij}$ = # of corpora lutea for j-th female in the i-th group

b. The Freeman-Tukey transformation is applied to the $Y_{ij}$ as follows:

$$f_{ij} = \sin^{-1}\sqrt{\frac{y_{ij}}{V_{ij}+1}} + \sin^{-1}\sqrt{\frac{y_{ij}+1}{V_{ij}+1}}$$

The t-test is then applied to the f's, comparing the test groups to the negative control. Let

$$\bar{f}_i = \frac{1}{n_i}\sum_{j=1}^{n_i} f_{ij}$$

$$s_i^2 = \sum_{j=1}^{n_i} (f_{ij} - \bar{f}_i)^2$$

where $n_i$ = # of pregnant females at dose level i.

Then $t = (\bar{f}_0 - \bar{f}_i)/\left[\frac{s_0^2 + s_i^2}{n_0 + n_i - 2}\left(\frac{1}{n_0} + \frac{1}{n_i}\right)\right]^{\frac{1}{2}}$

c. Regression analysis is used to determine whether the average number of preimplantation losses per female is related to the arithmetic or the log dose. The method is as used in Step 2 above substituting

$U_i$ = # of preimplantation losses for the i-th female.

5. Dead Implantations

The dead implants were evaluated by the same statistical techniques that were used in evaluating the total number of implantations.

Substitute

$u_{ij}$ = # of dead implants for j-th female in the i-th group in the equations in Step 2 above.

6. Proportion of Females with One or More Dead Implantations

The proportion of females with one or more dead implants is the number of females with dead implants/number of pregnant females. These proportions are analyzed by the same method used to analyze the fertility indices, i.e., by a chi-square test and Armitage's trend.

Substitute $n_i$ = # of pregnant females with one or more dead implants at dose level i and

$N_i$ = # of pregnant females at dose level i in Step 1 above.

Also a probit regression analysis is done using these proportions, $p_i$, to determine whether the probit of $p_i$ is linearly related to the log or arithmetic dose. The Biomedical Computer Program BMD03S is used to compute A and B and the $\chi^2$ statistic for the regression equations $y = A + B\,x$ and $y = A + B \log x$.

7. Proportion of Females with Two or More Dead Implantations

The proportion of females with two or more dead implantations is the number of females with two or more dead implants/number of pregnant females. The data are evaluated by the same method used for evaluating the proportion of females with one or more dead implants.

8. Dead Implants/Total Implants

Dead implants/total implants were computed for each female and transformed by way of the Freeman-Tukey arc-sine transformation prior to being evaluated by t-test to compare each treatment group and positive control to negative control.

Use $y_{ij}$ = # dead implants for j-th female in i-th group

$v_{ij}$ = # of total implants for j-th female in i-th group

in the equations in Step 4 above.